New Host-strains for the Lysogenic *Corynebacterium diphtheriae* PARK WILLIAMS No. 8 Strain

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SUMMARY

Investigation of the lysogenicity of seven *Corynebacterium diphtheriae* PW 8 variants revealed the spontaneous liberation, by these strains, of phage particles active against two *C. ulcerans* strains (9304 and 298 G) which are sensitive to *C. diphtheriae* *gravis* and *mitis* phages. By this means, phage particles with different host-range activity and morphology of plaques were obtained from the PW 8 strains. Further studies showed that some *C. diphtheriae* strains which possess the same sensitivity towards *gravis* and *mitis* phages showed the same spontaneous liberation of PW 8-carried phages. The newly obtained phages were able to convert to toxinogenesis both *C. ulcerans* and *C. diphtheriae* strains.

INTRODUCTION

During the course of lysotyping certain strains of *Corynebacterium diphtheriae*, received from Dr D. T. Simmons of the Commonwealth Serum Laboratories, Melbourne, Australia, we observed that some of the strains spontaneously liberated phage particles active against two *C. ulcerans* strains, 9304 and 298 G. The *C. ulcerans* strains were recently isolated in Rumania and were used as indicator strains for diphtheria phages. Since one of the lysogenic strains from Australia was labelled *intermedius* C.S.L. TORONTO (referred to as TORONTO), we assumed it to be the Toronto variant of the *C. diphtheriae* PW 8 strain and consequently tested it for toxigenicity in a static broth culture. The identity of the strain TORONTO was established by the production of diphtheria toxin of high potency (100 Lf per ml.) and demonstrating similar lysogenic capacity in all PW 8 collection variants existing in the Diphtheria Department of the 'Dr I. Cantacuzino' Institute. The strain isolated by Park & Williams (1896) and used for large-scale diphtheria toxin production in all countries is known to be different from most lysogenic toxinogenic *C. diphtheriae* strains since it carries a defective phage (Barksdale, Garmise & Horibata, 1960; Barksdale, Garmise & Rivera, 1961; Matsuda & Barksdale, 1966, 1967). Since our observation was in disagreement with the findings of others which stated the defective inducible nature of the PW 8-carried phage, a detailed study was begun to demonstrate more conclusively the spontaneous liberation of phage particles by the PW 8 strains. At the same time, we tried to analyse the relationship existing between PW 8 and *C. ulcerans* strains and to find, if possible, other receptive strains to confirm the spontaneous liberation of PW 8 phages.

METHODS

Corynebacteria strains. The following *Corynebacterium diphtheriae* PW 8 variants from the collection of the Diphtheria Department of the 'Dr I. Cantacuzino' Institute: the WEISSENSEE and DESSAU strains, received from the Weissensee Institute at Berlin; CN 2000, received from the Rijks Instituut vor de Volksgezondheid at Utrecht; MASSACHUSETTS and CN 2000 from the Human Institute at Budapest (the cultures labelled CN 2000 came originally from the Wellcome Research Laboratories, Beckenham, Kent, England, CN 2000 being their number for their original PW 8 strain); and the 474 strain received from the Pasteur Institute, Paris; the Australian strain TORONTO from the Commonwealth Serum Laboratories, Melbourne; the *C. ulcerans* strains were isolated in Rumania, 9304 (–) tox– from a healthy human carrier, and 298 G (–) tox– from a horse.

Other strains used were Freeman's *Corynebacterium diphtheriae mitis* strains nos. 770, 411, 444, 1174, 1180; the *C. diphtheriae mitis*-like strains nos. A 003, A 028, C 43, S 038 supplied by Dr N. B. Groman of the Department of Microbiology, University of Washington, Seattle; and the *C. belfanti* 1030 strain received from Dr H. Oehring of the Hygiene Institut, Friedrich Schiller Universität, Jena. The 9304 (W) tox+ strain was derived from the 9304 (–) tox– strain lysogenized with PW 8 phage.

All strains were investigated for their morphology, biochemical behaviour and *in vitro* toxin production according to the minimal requirements of the scheme for bacteriological diagnosis of *Corynebacterium diphtheriae* (Bulletin, 1965).

Detection of lysogeny. The method used for demonstrating liberation of phages was previously described by Saragea & Maximescu (1964). This method demonstrates phage particles, spontaneously liberated from the lysogenic strain in shake culture, by the spot method on agar plates of the sensitive indicator strain.

Toxinogenicity. Tests were performed on filtrates of Pope-Linggood broth cultures of the lysogenized strains: (1) in rabbit skin by the method of Fraser (1931); (2) by the subcutaneous inoculation of guinea-pigs; and (3) by *in vitro* inoculation of monkey kidney tissue cultures. Quantitative determination of large amounts of diphtheria toxin was done by the flocculation test of Ramon & Richou (1950).

The phage types of all strains were established according to the lysotyping scheme for *Corynebacterium diphtheriae* (Saragea & Maximescu, 1964, 1966).

RESULTS

Lysogenicity of PW 8 variants

The lysogenicity testing of the PW 8 variants (WEISSENSEE, DESSAU, CN 2000 from Utrecht, MASSACHUSETTS and CN 2000 from Budapest, 474, and TORONTO), carried out with stock cultures of each strain, showed spontaneous liberation of phages of *Corynebacterium ulcerans* strains 9304 (–) tox– and 298 G (–) tox–. Previous tests performed with *C. ulcerans* strains had established that they were non-lysogenic and non-diphtheria toxin producers. Appearances of lysis with punctiform plaques were noted. By multiplication of the WEISSENSEE, CN 2000 Utrecht, and TORONTO lysates on the 9304 (–) tox– *C. ulcerans* strain, the three phage preparations, 'W', 'U', 'T', with confluent lysis were obtained. The new phage preparations lysed mainly the *C. ulcerans* strains, but had also a weaker activity (a few plaques) on Freeman's strain *C. diphtheriae* 770 (Table 1).

Considering that all PW 8 variants used in this study were stock cultures and that in general among populations of the high toxinogenic PW 8 strain mutants occur displaying different lysogenic qualities (Barksdale *et al.* 1961), we tried to repeat the lysogenicity test, starting from isolated colonies of the WEISSENSEE and CN 2000 variants. The tests performed show similar results (Table 2).

Table 1. *Activity of phage particles spontaneously released by WEISSENSEE, CN 2000 and TORONTO strains*

Sensitive strains	PW 8, WEISSENSEE		PW 8, CN 2000 Utrecht		TORONTO	
	Original lysate*	Phage 'W'†	Original lysate*	Phage 'U'†	Original lysate*	Phage 'T'†
9304	Scanty punctiform plaques	OL	1+s	OL	1+s	OL
298 G	Scanty punctiform plaques	OL	1+s	OL	1+s	OL
770	—	Isolated, normal-sized plaques	.	Isolated, normal-sized plaques	.	Isolated, normal-sized plaques

* An overnight shake culture of the stock PW 8 strains. 1+s = > 50 small, punctiform plaques.

† Phages 'W', 'U' and 'T' originated from the multiplication of the spontaneously released pw 8 phage particles on the 9304 (–) tox– *C. ulcerans* strain. OL = opaque lysis.

Table 2. *The lysogenic state of some isolated colonies from the highly toxinogenic WEISSENSEE and CN 2000 strains*

Strain	Colony no.	Diphtheria toxin, produced in static culture (Lf/ml.)	Type of lysis obtained on <i>C. ulcerans</i> strains 9304 and 298 G
PW 8, WEISSENSEE	112	100	Scanty punctiform plaques
	113	90	Scanty punctiform plaques
	114	70	Scanty punctiform plaques
PW 8, CN 2000	115	95	1+s*
	116	95	1+s*
	117	100	1+s*

* 1+s = > 50 small punctiform plaques.

Starting from isolated plaques of the 'W' phage on plates with different host strains (9304, 770 and 411), three kinds of phage preparations with different spectra of activity were obtained (Table 3).

The similarity of the host-range activity of the W/411 and Freeman's B phage is remarkable. Attention is also drawn to (1) the minute plaques of the W/9304 phage obtained on *Corynebacterium ulcerans* strains (Pl. 1, fig. 1a, b); (2) the normal size plaques of the W/770 and W/411 phages; and (3) the different behaviour towards pw 8 phages of the 770 strain among Freeman's other strains (Table 3).

Table 3. *Host range of PW 8 phages as compared with Freeman's B phage*

Indicator strains	Bacteriophage			
	W/9304*	W/770*	W/411*	Freeman's phage B
411	.	.	CL	CL
444	.	.	CL	CL
1174	.	.	CL	CL
1180	.	.	CL	CL
770	1+n	OL	CL	CL
9304	OL	1+n	1+n	1+n
298 G	OL	1+n	1+n	1+n

* Each of the PW 8 phages was obtained by multiplication of a single titre plaque from the original lysate of the WEISSENSEE strain seeded on the growth of the 9304, 770 and 411 strains.

CL, Clear confluent lysis; OL, opaque confluent lysis; 1+n, > 50 normal-sized plaques.

Lysogenic conversion by PW 8 phages

The lysogenization by PW 8 phages of the 9304 (–) tox – *Corynebacterium ulcerans* and 770 (–) tox – *C. diphtheriae mitis* strains demonstrates their converting capacity. Single colonies isolated from the lysis area on solid medium (for each phage, 20 colonies from the respective strain) were tested in parallel with an equal number of colonies from the original strain. The results showed different rates of conversion (20/20) for the *C. diphtheriae* 770 (–) tox – and (5/20) *C. ulcerans* 9304 (–) tox – strains. The conversion of the *C. ulcerans* 9304 (–) tox – strain caused us to analyse the change produced in the lysogenized population in greater detail. For demonstration of the newly formed diphtheria toxin by the lysogenized *C. ulcerans* (9304 (W) tox+) cultures, the *in vitro* Elek precipitation test, the *in vivo* test on rabbit skin (Pl. 1, fig. 2a, b), the *in vivo* guinea-pig inoculation test and *in vitro* tissue culture inoculation (Pl. 2 fig. 3a, b) were set up. The results, which are presented in Table 4, show the identity of the newly produced toxin with diphtheria toxin.

The lysogenized diphtheria-toxinogenic 9304 (W) tox+ strain did not change the morphological, biochemical and biological behaviour of a *C. ulcerans* strain, but became resistant to lysis by PW 8 phages.

The phage liberated by the 9304 (W) tox+ strain had the same capacity to convert the original 9304 (–) tox – strain to toxinogenicity as the original phages released by PW 8 strains.

Other strains receptive for the spontaneously released PW 8 phage particles

In view of the fact that *Corynebacterium ulcerans* and *C. diphtheriae* strains are closely related (Petrie & McClean, 1934; Jebb, 1948; Jebb & Martin, 1965), we considered that the receptivity of the two *C. ulcerans* strains for PW 8 phages is not due to their specificity as *C. ulcerans* strains, but to their wide receptivity for diphtheria *gravis* and *mitis* phages. For this reason, we selected from the Diphtheria Department's collection some strains which presented this feature (Table 5): *C. diphtheriae mitis*-like strains nos. A 003, A 028, C 43, S 038, and *C. belfanti* strain no. 1030. All these strains were receptive for spontaneously released PW 8 phages (Pl. 2, fig. 4).

Table 4. Tests to demonstrate the identity of the toxin produced by the lysogenized *Corynebacterium ulcerans* strain with diphtheria toxin

Strain and colony no.	Sensitivity towards pw 8 phages	Lysogenicity against 9304 (—) tox—original strain	In vivo tests				In vitro tests				
			Skin test on rabbit (Fraser's method)	Guinea-pig subcutaneous inoculation		Monkey kidney tissue culture inoculation		Elek-Ouchterlony test‡			
				Test*	Control with 5000 i.u. diphtheria antitoxin	Test†	Control with 2000 i.u. diphtheria antitoxin			Test	Control with 5 i.u. diphtheria antitoxin
9304 (W) tox + lysogenized strain	1	+	Filtrate i.N.	i.	+	—	++	+	+		
	2	+	Culture i.n.N.	i.n.	+	++	±	—	+		
	3	+	Filtrate i.N.	i.	+	—	++	—	+		
		+	Culture i.n.N.	i.n.	+	++	±	—	+		
	19	+	Filtrate i.N.	i.	+	—	++	+	+		
9304 (—) tox—control strain pw 8 WEISSENSEE strain	20	+	Filtrate i.N.	i.	+	—	++	+	+		
		—	Filtrate i.	i.	+	—	++	—	—		
		+	Culture i.n.	i.n.	+	++	—	—	—		
		—	Filtrate N.	—	+	—	++	+	+		
		+	Culture N.	—	+	—	++	+	+		

* i = infiltration (activity of *C. ulcerans* strains positive for filtrates or cultures); n = haemorrhagic necrosis (activity of *C. ulcerans* cultures); N = necrosis produced by diphtheria toxin (in filtrates and cultures).

† + = guinea-pigs die within 24–48 hr with gelatinous oedema at the site of inoculation and haemorrhagic adrenal gland (symptoms typical of diphtheria toxin neutralized with diphtheria antitoxin); ++ = guinea-pigs die within 24–48 hr with haemorrhagic gelatinous oedema at the site of inoculation (typical of *C. ulcerans* not neutralized by diphtheria antitoxin) and also haemorrhagic adrenal glands (typical of diphtheria toxin); +++ = guinea-pigs die within 48–96 hr with haemorrhagic gelatinous oedema at the site of inoculation (typical of *C. ulcerans*).

‡ Done on a Loeffler culture of each strain.

DISCUSSION

The synthesis of diphtheria toxin occurs through a mechanism controlled by the gene of a bacteriophage which converts the receptive host to lysogeny and at the same time confers upon it the tox + character (Freeman, 1951; Barksdale, 1959). In general, toxinogenesis and lysogenicity are correlated and the presence of the tox + character implies carriage of a bacteriophage, the detection of which is dependent on the availability of a host strain. This fact became obvious to us on numerous occasions when apparently non-lysogenic, toxinogenic *Corynebacterium diphtheriae* strains liberated a bacteriophage which could be detected only when tested on a larger set of sensitive strains. By this means the prophage is not difficult to demonstrate. Numerous studies on the mechanism of diphtheria toxin production have focused attention on the behaviour of the highly toxinogenic PW 8 strain. Such studies demonstrated that this strain differs from most lysogenic *C. diphtheriae* strains by the defective phage it carries, which harbours a genetic block that prevents the formation of mature phage (Barksdale, 1959); for this reason, although generally phage liberation and toxin synthesis are closely correlated, this correlation could not be established with the PW 8 strain (Matsuda & Barksdale, 1966). In the present work, using two *C. ulcerans* and some *C. diphtheriae* strains which present the same wide receptivity to diphtheria *gravis* and *mitis* phages, we were able to demonstrate the spontaneous liberation of phage particles by PW 8 strains, with different host range activity, amongst which one was similar to Freeman's phage B. Thus our findings agree with those of Miller, Pappenheimer & Doolittle (1966), who demonstrated in the PW 8 lysate a blocking antigen for anti-B phage sera which caused them to state: 'It is at least as likely that the PW 8 strain carries a normal phage genome. . .', and with those of Mathews, Miller & Pappenheimer (1966), who showed by electron-microscope photographs a phage resembling phage B in PW 8 lysates. In the present investigation, using suitable host strains, the presence of prophage was easily demonstrated.

Thus the results of this investigation open some new aspects of the PW 8 strain: PW 8 strains are not defective lysogenically, but spontaneously liberate phage particles with different host-range activity; the host strains which display spontaneous liberation of PW 8 phages (*Corynebacterium ulcerans*, *C. diphtheriae mitis*-like, and *C. belfanti*) are strains sensitive for a large number of diphtheria *gravis* and *mitis* phages; phages released by PW 8 strains convert to diphtheria toxinogenesis sensitive strains of both *C. ulcerans* and *C. diphtheriae*; all PW 8 variants are resistant to a large number of *gravis*, *intermedius* and *mitis* phages.

The possibility of demonstrating by means of the new host strains the spontaneous release of PW 8-carried phages, and the classification of the PW 8 strain, according to our conception, as an atypical *gravis* type (McLeod, 1943) opens new prospects for the study of phage-host relationships and bacteriophage-directed synthesis of diphtheria toxin—as well as for the 'iron effect' (Matsuda & Barksdale, 1967) in this highly toxinogenic strain.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. Plaques produced by pw 8 phages on *Corynebacterium* strains. *a*, Minute plaques produced on 9304 (—) tox— *Corynebacterium ulcerans* strain. *b*, Normal size plaques produced on *C. diphtheriae* strains. Magnification of both *a* and *b* $\times 20$.

Fig. 2. Skin tests in rabbits of toxic filtrates with and without diphtheria antitoxin. *a*: 1, *Corynebacterium ulcerans* filtrate from 9304 (—) tox—; 2 and 3, filtrate from 9304 (W) tox+ lysogenized strain; 4, pw 8 diphtheria toxin. *b*, The filtrates, in the same order, with diphtheria antitoxin. It will be seen that the pw 8 toxin is neutralized in 4, and also the necrosis in 2 and 3.

Fig. 1

Fig. 2

Fig. 3

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PLATE 2

Fig. 3. Cytopathogenic effect of filtrate on monkey tissue. *a*, Cytopathogenic effect of 9304 (W) tox + filtrate on monkey tissue culture. *b*, Similar tissue culture in which toxic effect of 9304 (W) tox + filtrate has been neutralized with diphtheria antitoxin.

Fig. 4. Lysis produced by spontaneously released PW 8 phages by (1) WEISSENSEE, (2) CN 2000 Budapest, (3) MASSACHUSETTS, (4) WEISSENSEE, (5) CN 2000 Utrecht, (6) TORONTO strains seeded on a growth of *Corynebacterium belfanti* strain 1030.